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The Influence of HDL Oxidation on Cholesterol Efflux from Mammalian Cells

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Istud, quod tu summum putas, gradus est… (Seneca)

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1 Introduction

Cardiovascular diseases (CVDs) are worldwide medical and public health problems that together constitute the leading cause of death in the Western world. The majority of CVDs are attributable to atherosclerosis, a disease associated with inflammatory events and increased oxidative stress (Lloyd Jones, 2009).

A number of studies revealed an inverse correlation of High density lipoprotein (HDL) cholesterol (C) plasma levels with cardiovascular risks (for recent reviews see Von Eckardstein 2010; Lewington, 2007). HDL exhibits potentially anti-atherogenic properties attributed to its ability to participate in a process termed reverse cholesterol transport (RCT). In this scenario HDL transports cellular cholesterol from peripheral tissues to the liver and intestine for excretion in the bile and feces (Tall, 2008). The scavenger receptor class B type I (SR-BI) mediates the last step in RCT, the delivery of HDL cholesteryl esters (CE) to the liver, mainly by selective CE uptake (Krieger, 1999).

It has been reported that HDL looses its cardioprotective properties through oxidative modifications generating dysfunctional HDL. ApoA-1, the most abundant protein of HDL, is assumed to be the major target for oxidative modifications, indicated by reactive oxygen species (ROS) and myeloperoxidase, both present in atherosclerotic lesions as a response to inflammatory processes in the progression of atherosclerosis (Shao, 2006 & 2010). The underlying mechanisms remain poorly understood, but it is assumed that dysfunctional HDL may result in different cholesterol transfer capacities and alterations in RCT. Moreover, SR-BI may be involved in the binding of modified HDL (Marsche, 2002).

The objectives of this diploma thesis were to characterize whether oxidative modifications of HDL influence cholesterol efflux from mammalian cells. Furthermore, we attempted to analyze if these modifications alter cellular binding and uptake compared to native HDL.

2 Literature Overview

2.1 Atherosclerosis

2.1.1 Epidemiology

Cardiovascular diseases (CVD) are the leading cause of death of middle-aged and elderly people in the western world. Atherosclerosis is the underlying cause for the development of CVD. In the USA about 80 million people (or 36% of the whole population) obtain existing CVD; 12 million of these individuals in the USA are affected by atherosclerosis with a tendency to rise. Similar trends can be observed in Europe (Lloyd Jones, 2009; Cooke, 2010).
Efficacious prevention of cardiovascular diseases includes the treatment of the most important risk factors such as cigarette smoking, obesity, diabetes and elevated cholesterol levels, especially increased LDL-cholesterol levels (Tamada, 2010). For effective cardiovascular prevention the detection and recognition of early stages of atherosclerosis is very important. Additionally, the role of fibrinogen and highly sensitive C-reactive protein (hs-CRP), to serve as new markers in the early detection of atherosclerosis, is discussed (Corrado, 2010).

2.1.2 Pathogenesis

Atherosclerosis is an inflammatory disease, characterized by endothelial dysfunction, vascular inflammation and excess cholesterol within the intima of the vessel wall. The development of atherosclerotic lesions is the initial step in the pathogenesis of atherosclerosis (Libby, 2009).

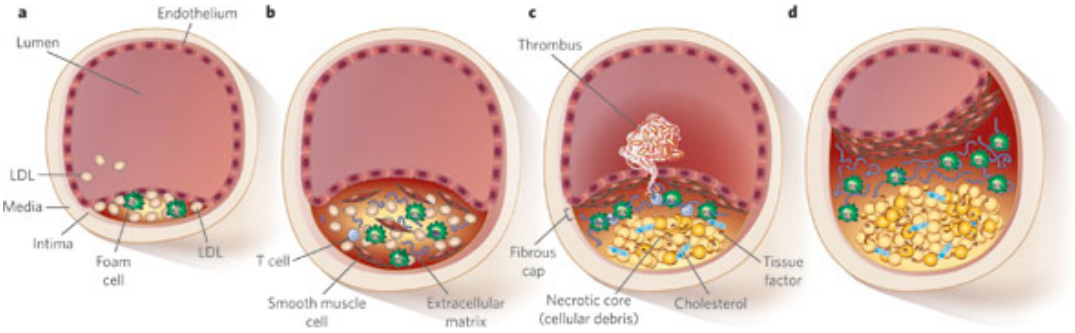
Functional cell-monolayers within the artery exhibit homeostatic endothelial regulation mechanisms to protect against detrimental effects which can damage them. For example, healthy vascular endothelial cells combat thrombosis, express enzymes, such as superoxide dismutase, to protect against reactive oxygen species and produce vasodilator molecules to resist blood flow.
Exposed to proatherogenic factors, namely increased cholesterol levels, modified lipoproteins, such as oxidized LDL, or disturbed blood flow, the cells of an artery became dysfunctional (Libby, 2010). As a response endothelial cells

express leukocyte adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and leukocytes then enter the endothelial barrier. This is followed by attachment of other blood cells and cytokine release of the intima (Goldberg, 2009). This process is enhanced by monocytical proliferation mediated by monocyte-colony stimulating factor (M-CSF). Macrophages internalize intracellular cholesterol derived from lipoproteins within the developed plaques in the artery (Hansson, 2005). This results in foam cell formation and is a further characteristic in the progression of atherosclerosis and impacts early stages of atherogenesis as well as late lesion development (Boyle, 2005).

Monocyte recruitment is aided by endothelial expression of inflammatory signals and activation in the cytokine cascade. The cytokine cascade activation leads to an increased release of interleukin-6 (IL-6), interleukin-1 (IL-1), tumor necrosis factor α (TNFα), C-reactive protein (CRP) and some more (Goldberg, 2009).

Figure 1: Progression of Atherosclerosis (Rader, 2008)

The development of fatty streaks to occlusions is shown. For further description, see text.



Atherosclerosis occurs at sites of an artery where laminar flow is disrupted. A lesion is initiated as a fatty streak (Fig. 1a), can develop into an intermediate lesion (Fig. 1b), further progress into a lesion that is vulnerable to rupture (Fig. 1c) and, finally, into an advanced obstructive lesion (Fig. 1d). The fatty streaks are thought to be the initial status a lesion leading to the development of complex atherosclerosis. Plaque, within an artery can rupture, resulting in the exposure of thrombogenic material and the formation of a thrombus in the lumen. Finally, the developed thrombus can narrow or completely block the

artery, leading to myocardial infarction, stroke and peripheral artery occlusive disease (PAOD; Rader, 2008).

Inflammatory events and increased cell immune response potentially account for the development of atherosclerosis and in general for cardiovascular diseases (Corrado, 2010). As mentioned above, lipoproteins are critically involved in these processes.

2.2 Lipoproteins

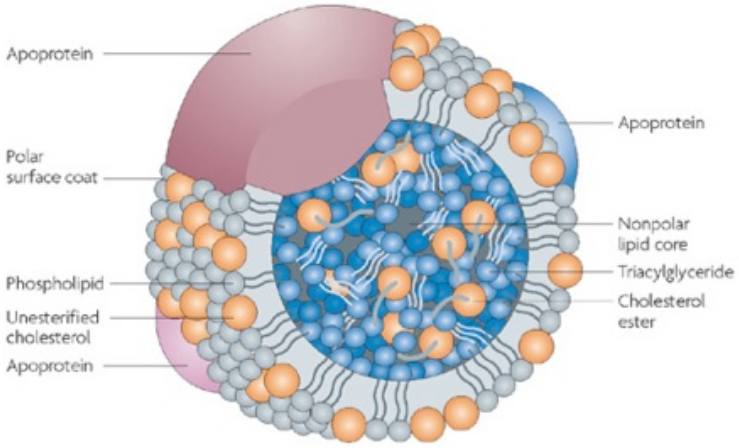
2.2.1 Lipoprotein Metabolism

Lipoproteins transport fatty lipids in the blood.

Lipoproteins are classified according to their specific densities and molecular sizes into high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons. Besides, these classes of lipoproteins differ in physiological function, lipid composition and apolipoprotein content (Olofsson, 2009). Lipoproteins consist of a core of neutral lipids, such as triglycerides and cholesteryl esters, surrounded by an amphiphilic shell of phospholipids, free cholesterol and proteins, so-called apolipoproteins (Fig. 2; Wasan, 2008).

Figure 2 Composition of Lipoproteins (Wasan, 2008)

Lipoproteins consist of a core of neutral lipids, surrounded by an amphiphilic shell of phospholipids, free cholesterol and proteins, so-called apolipoproteins.

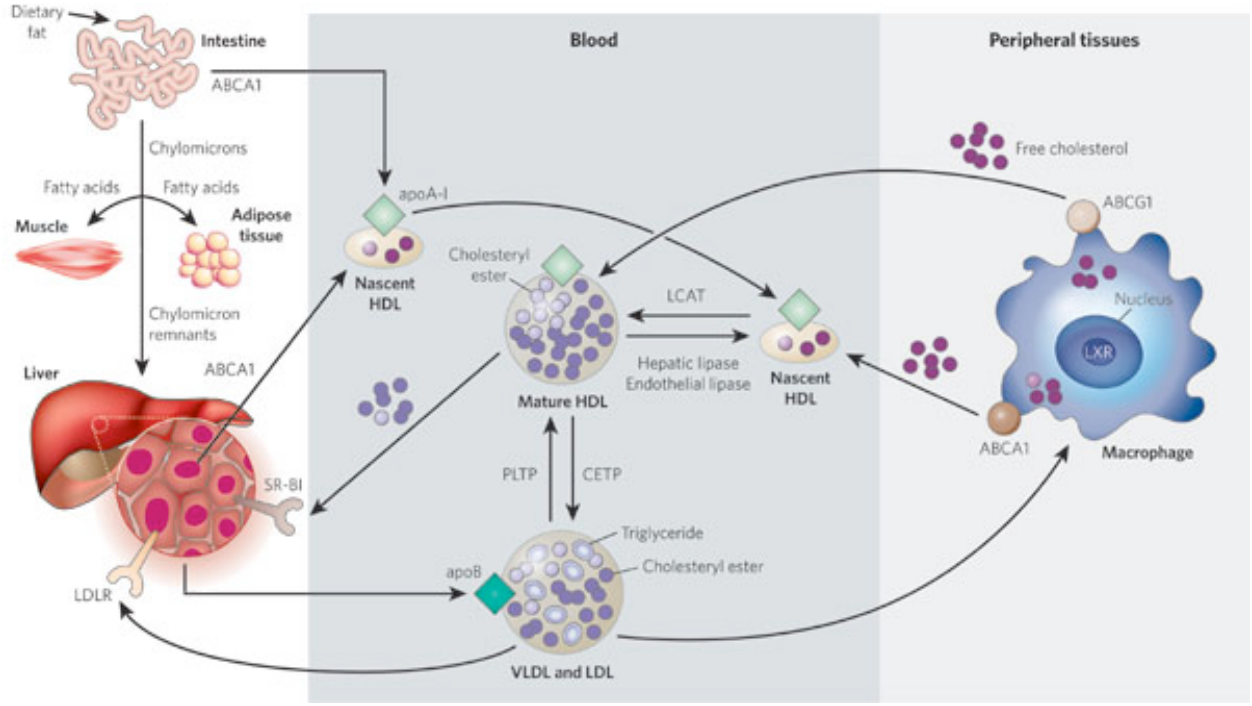


The lipoprotein metabolism follows two main pathways: the exogenous pathway, where lipoproteins are mainly composed of dietary lipids and the endogenous pathway, where lipoproteins originate in the liver (Wasan, 2008).

After oral ingestion and digestion of food, dietary fat is absorbed by the intestine and packaged into chylomicrons (Fig. 3); in this stage they are termed nascent chylomicrons and contain apoB-48. Chylomicrons enter the circulation and acquire apoC-2 and apoE from HDL. This process leads to the maturation of nascent chylomicrons in the blood. Chylomicrons are transported by blood to muscle and adipose tissues and within these tissues the enzyme lipoprotein lipase (LPL) breaks down chylomicrons. LPL catalyzes the hydrolysis of triglycerides in chylomicrons to transfer fatty acids to tissues (Daniels, 2009). Additionally, this process generates non-esterified fatty acids in plasma and remodels chylomicrons into chylomicron remnants (CR). CR are taken up by the liver and CR lipids are hydrolyzed for VLDL synthesis (Rigotti, 2003). Within hepatocytes lipids of CR are used for apoB lipidation, which is then secreted as VLDL. In the circulation VLDL exchanges apoC-2 and apoE with HDL. VLDLs deliver free fatty acids to adipose and muscle tissue and activate LPL. Like chylomicrons, VLDL triglycerides are hydrolyzed by LPL to rise smaller intermediate density lipoproteins (IDL) particles. IDL are either removed from circulation by hepatic endocytosis, which includes receptors, such as the LDLR, or, through the action of cholesteryl ester transfer protein (CETP) and hepatic lipase, further converted to low density lipoproteins (LDL; Maxfield, 2002). LDLs are the major carriers of cholesterol and cholesteryl esters in humans. LDL is removed from circulation by LDL receptor mediated endocytosis, a process in which LDL is internalized and delivered to lysosomes for degradation (Brown, 1986).

Figure 3. Lipoprotein Metabolism (Rader, 2008)

Lipoprotein metabolism has a key role in atherogenesis. It involves the transport of lipids, particularly cholesterol and triglycerides, in the blood mediated by lipoproteins. For further description, see text.



2.2.2 High Density Lipoprotein (HDL)

Anti-Atherogenity of HDL

There is evidence for an inverse association between plasma high density lipoprotein cholesterol (HDL-C) concentrations and the risk of cardiovascular disease. HDL is thought to protect against the future development of CVD related to mechanisms that have a potentially anti-inflammatory capacity (Cooney, 2009).

The major antiatherogenic function of HDL is associated with its ability to mediate cholesterol efflux from cells in a process termed reverse cholesterol transport (Rothblat, 2010). A key-role of HDL in vascular protection is the removal of cholesterol from lipid-laden macrophages within the vessel walls (Vergeer, 2010).

HDL Composition

High density lipoprotein is the smallest lipoprotein with a density in the range of 1,063-1,21 g/ml and contains the least amount of lipids (Rader, 2008). The shape of HDL particles ranges from discoidal to spherical with different subclasses of HDLs, which vary in lipid and protein composition, density and electrophoretic mobility (Lund-Katz, 2003).

On the basis of electrophoretic mobility, HDL can be separated into two main subgroups, namely α-HDL and β-HDL. In plasma, most HDL can be found as α-HDL whereas β-HDL represents only approximately 5-15% (Rothblat, 2010).

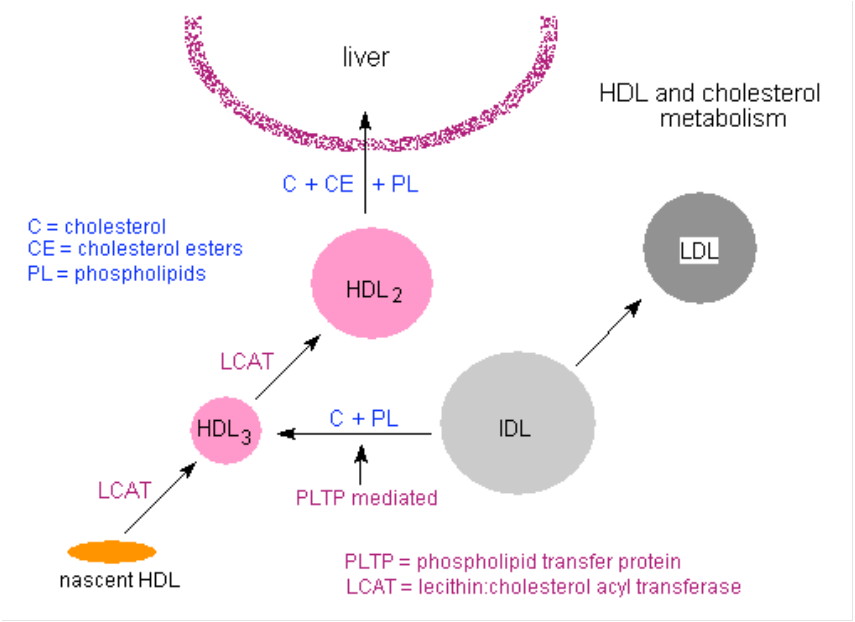
The major component of both subclasses of HDL is the apoA-1 protein. ApoA-1 is supposed to activate the enzyme lecithin:cholesterol acetyl transferase (LCAT) which is responsible for the esterification of plasma cholesterol (Rader, 2009).

HDL is secreted by the liver and intestine in form of nascent HDL, containing apoA-1 and free cholesterol (FC). Nascent HDL interacts with peripheral cells, such as macrophages, to remove excess free cholesterol (Liu, 2003; Duong, 2006). This process is mediated by the ATP-binding cassette A1 (ABCA 1) gene. Nascent HDL is converted into mature HDL (Fig. 4), which has a high amount of cholesteryl esters, which are synthesized from free cholesterol and fatty acids by the enzyme LCAT (Duong, 2006).

Figure 4. Maturation of High Density Lipoprotein

(http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm; 18.06.2010)

Nascent HDL is converted into mature HDL, mediated by the enzyme lecithin cholesterol acetyl transferase (LCAT) which requires apoA-1 for activation.



2.2.3 Oxidized Phospholipids and Dysfunctional HDL

Oxidation of Phospholipids is one of the hallmarks in the pathogenesis of atherosclerosis. Oxidized phospholipids (oxPLs) are associated with lipoproteins in the circulation and, besides activating inflammatory signals, oxidized phospholipids can inhibit the uptake of lipids associated with HDL. Additionally, the cholesterol transport mediated by HDL can be impeded by oxPL (Fu, 2008). The levels of oxPL in circulation increase in chronic and acute pathologic events, namely hyperlipidemia and atherosclerosis (Fu, 2008; Deigner, 2008).

Phospholipids associated with lipoproteins or cell membranes are the major source for oxPLs. oxPLs are generated by lipid peroxidation induced by an enhanced generation of reactive oxygen species (ROS) or decreased antioxidant defense (Fu, 2008). Once they are oxidatively modified they undergo a number of pathways inducing inflammatory signals, such as IL-8 and others, and cytotoxic events. Phospholipids containing polyunsaturated fatty

acids are particularly susceptible for modification by ROS (Deigner, 2008). Important biologically active PLs, generated by oxidation processes, include 1-palmitoyl-2-(5-oxovaleroycl)-sn-glycero-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-phosphocholine (PGPC) and 1-palmitoyl-2-(5,6-epoxy-cyclopentenone)-sn-glycero-3-phosphocholine (PECPC). POVPCs and PGPCs are generated through oxidatively induced truncation of the sn-2 residues of fatty acids, whereas PECPC development is based on the addition of oxygen atoms to the sn-2 fatty acid residues (Fu, 2008).

oxPLs associated with HDL serve as ligands for CD36 as well as SR-BI. Studies demonstrated that oxPLs prevent HDL binding to SR-BI because of the close proximity of the structure of both receptors (Gao, 2010). The interaction between oxPLs and the SR-BI receptor may indicate an inhibition of HDL derived lipids, which leads to indifferent cholesterol transfer capacities. Moreover, selective uptake of cholesteryl esters seems to be inhibited by increased levels of oxPLs (Ashraf, 2008).

In addition to oxPLs, modifications of apoA-1, the major protein of HDL, may also implicate oxidative generation of dysfunctional HDL attributed to alterations in lipid transfer and selective cholesteryl ester (CE) uptake derived from HDL (Ashraf, 2008).

Reactive carbonyls, such as malondialdehyde (MDA), develop due to lipid peroxidation and have been found in atherosclerotic lesions *in vivo*. ApoA-1 seems to serve as a potential target for modifications by reactive carbonyls (Shao, 2010).

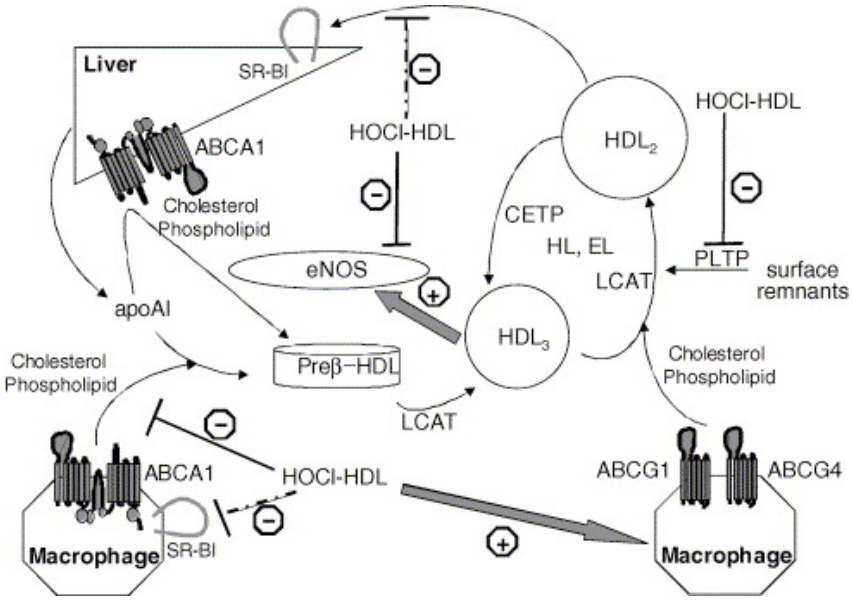
Especially lysine residues within apoA-1 underlie changes in their chemical structure. Modification of apoA-1 by MDA forms cross-links between different lysine residues and this may affect the conformational adaptability of apoA-1 (Shao, 2006). This results in a reduced ability of apoA-1 to interfere with ABCA1 (Fig. 5), a major receptor for apoA-1 associated lipids in reverse cholesterol transport (RCT). Furthermore, MDA modified apoA-1 within reconstituted HDL looses its ability to activate lecithin:cholesterol acyltransferase (LCAT), an important enzyme in RCT and HDL maturation (Shao, 2010).

MDA mediated oxidation also severely limited the ability of apoA-1 to exchange between HDL-associated lipid-free and lipid-poor states, a keyrole mechanism in the progression of atherosclerosis (Cavigiolio, 2010). Beyond these findings,

it was reported that dysfunctional HDL is associated with decreased paraoxonase-1 (PON1) activity. PON1 is an esterase that is found in circulation bound to HDL and it is thought to degrade oxidized phospholipids in lipoproteins (Marsillach, 2010). Alterations in PON1 levels in circulation have been found associated with diseases involving oxidative stress, such as atherosclerosis (Mackness, 1995).

Figure 5. HDL modified with hypochlorite is able to interfere with RCT (Malle, 2005).

Overview of the HDL metabolism: the dashed lines show possible interaction pathways for HOCl modified HDL in reverse cholesterol transport.



2.3 Cellular Cholesterol Homeostasis

Cholesterol is an essential component of the plasma membrane of mammalian cells. It maintains the barrier function between cells and environment, modulates fluidity, and is also the precursor for steroid hormone synthesis and bile acids. The cellular supply of cholesterol can be maintained by three defined mechanisms: synthesis of cholesterol, uptake and cholesterol efflux. The homeostasis of cellular cholesterol content is essential for optimal cell viability and function (Rader, 2008).

Cholesterol is synthesized in the endoplasmatic reticulum (ER) under the control of the enzyme acetyl-CoA by 3-hydroxy-3-methylglutaryl coenzyme A

(HMG-CoA) reductase. Additionally to the biosynthesis, cholesterol levels are controlled by endocytosis of lipoproteins.

Excess cholesterol derived from lipoproteins is stored as cholesteryl esters within lipid droplets. The storage of cholesterol as cholesteryl esters is controlled by the esterifying enzyme acyl CoA:cholesterol acyltransferase (ACAT). Cholesteryl esters from apo-B/E containing lipoproteins are taken up via the LDL receptor (LDLR). After endocytosis cholesteryl esters are hydrolyzed in endosomes and lysosomes by acidic cholesteryl ester hydrolases (Wang, 2005). Cholesterol levels and intracellular cholesterol content are regulated by the LDL receptor and HMG-CoA reductase (Brown, 1986). The transcription levels of the LDLR and HMG-CoA genes are controlled by transcription factors including sterol responsive element binding proteins (SREBPs). SREBPs are sensors for cholesterol and regulate cholesterol homeostasis by controlling de novo synthesis via HMG-CoA and cholesterol uptake via the LDLR. Additionally, the nuclear transcription factors liver X receptors (LXRs) regulate the expression of ABCA1 and ABCG1, both of them involved in cholesterol efflux from cells (Brown, 1986; Eberle, 2004).

2.3.1 Reverse Cholesterol Transport and Cholesterol Efflux from Cells

Reverse cholesterol transport (RCT) is a process described to remove excess cholesterol from peripheral tissues for disposal by the liver and the bile.
Peripheral tissues dispose cholesterol by effluxing to extracellular receptors, such as lipoproteins. ApoA-1, the mayor protein of HDL, serves as an acceptor for cellular cholesterol in its lipid-poor form (Rader, 2008).

Cholesterol efflux follows different pathways:
Efflux to lipid-poor apolipoproteins, such as apoA-1; this pathway is mainly mediated by ABCA1.
Efflux to mature HDL particles is promoted by ABCG1 and scavenger receptor class B type I (SR-BI; Rader, 2008).

Lipid -poor apoA-1, secreted from the liver, acquires free cholesterol, released from macrophages or other cells from peripheral tissues, via the ABCA1 pathway. Afterwards, free cholesterol, associated with nascent HDL, is converted into cholesteryl esters by lecithin:cholesterol acyltransferase (LCAT). In this process HDL is remodeled to mature HDL containing cholesteryl esters. Mature HDL transports its cholesterol to the liver for disposal and bile-secretion or steroid hormone synthesis. The transport of HDL-CE to the liver follows two main pathways. Selective uptake of HDL-CE mediated by SR-BI or by triglyceride-rich lipoproteins, such as LDL and VLDL, via the LDL receptor (LDLR; Tall, 2008; Brown, 1986). Selective uptake is considered as a non-endocytotic mechanism where lipoproteins bind with high affinity to SR-BI for lipid transfer. Afterwards, lipid-depleted lipoproteins are released from cells and re-enter circulation. Additionally, CE derived from HDL is transferred to LDL and VLDL by CE transfer protein (CETP) and subsequently removed from the circulation by endocytosis via the LDL-receptor (LDLR; Fluiter, 1997; Rhainds, 2002).

2.3.2 Important Receptors involved in Reverse Cholesterol Transport and Cholesterol Efflux from Cells

Scavenger Receptor class B type I (SR-BI)

SR-BI is described as a multi-ligand receptor, localized on the surface of cells, and belongs to the scavenger receptor CD36 superfamily. It is a 509-amino acid, 82 kDa glycoprotein containing a large extracellular domain and two transmembrane domains with short cytoplasmic amino- and carboxyl-terminal tails (for a review see: Murphy, 2005). SR-BI is expressed in tissues with crucial roles in cholesterol metabolism, namely the liver and steroidogenic tissues with the highest mass of SR-BI protein found in the liver, specifically, in parenchymal hepatic cells. These tissues exhibit the highest levels of selective uptake of cholesteryl esters derived from HDL (Krieger, 1999; Fluiter, 1998).

SR-BI was first identified as a receptor for oxidized LDL, acetylated LDL and native LDL (Krieger, 1994). Later, Acton et al. showed that SR-BI mediates selective uptake of HDL derived cholesteryl esters (CE) (Acton, 1996). Moreover, SR-BI is reported to serve as a receptor for oxidized HDL, for example, after oxidation by myeloperoxidase-generated hypochlorite (Marsche, 2002). SR-BI mediates bidirectional exchange of lipids, which is implicated as selective uptake of CE and FC from lipoproteins and efflux of FC to lipoproteins (Stangl, 1998; Swarnakar, 1998). Selective uptake of CE supplies cholesterol to liver and steroidogenic tissues and further for secretion by bile and for steroid hormone synthesis (Rhainds, 2004). In this process SR-BI promotes hepatic uptake of HDL (both esterified and unesterified) considered as a non-endocytotic mechanism, contrary to the LDLR mediated endocytosis. Lipoproteins bind with high affinity to SR-BI and after lipid transfer, lipid-depleted lipoproteins are released from cells and re-enters circulation. Multiple studies utilizing SR-BI knock out mice models indicate that HDL-CE selective uptake is diminished compared to wild type mice. These results further suggest an important role of SR-BI in reverse cholesterol transport (Rigotti, 2003).

In addition to selective uptake, HDL holo-particle uptake was shown to be mediated by SR-BI. This pathway, which includes HDL endocytosis and resecretion, is discussed as an alternative path to maintain cellular cholesterol

homeostasis in cases where cholesterol metabolism is disturbed (Pagler 2006 & 2007; Röhrl 2010).

ATP- binding cassette (ABC) transporters ABCA1 and ABCG1

ABC transporters are transmembrane proteins that utilize adenosine triphosphate (ATP) hydrolysis to transport various substrates, such as lipids and sterols, across extra- and intracellular membranes (Tall, 2008).

The expression of ABCA1 and ABCG1 is mainly regulated by liver X receptors (LXRs), ligand-activated transcription factors that belong to the nuclear receptor super family. In this process LXRs renders mutual compensation in the activities of both receptors (Tall, 2008). ABCA1 promotes cholesterol efflux to lipid-poor apoA 1 in a process that involves the direct binding of apoA-1 to the transporter (Oram, 2000). In contrast, ABCG1 promotes cholesterol efflux to a variety of lipoprotein particles, such as HDL, LDL and phospholipid-vesicles, but does not seem to bind lipoprotein particles (Wang, 2006).

To point out the importance of ABCA1, people with mutations or deficiency of ABCA1 suffer from low HDL cholesterol levels and increased accumulation of cholesterol in various tissues. This syndrome is termed Tangier disease (Rust, 1999). Furthermore, a set of studies suggest major roles of ABCA1 and ABCG1 in cholesterol efflux from macrophages to HDL. For example, mice with a combined deficiency of both receptors revealed an increase of cholesterol accumulation in form of cholesteryl esters as well as foam cell accumulation in various organs compared to wild type mice (Yvan-Charvet, 2007). Up-regulation of ABCA1 and ABCG1 transporters is thought to be a beneficial target for therapeutic strategies in cardiovascular diseases (Trogan, 2006).

3 Material and Methods

3.1 Tissue Culture

3.1.1 Cell lines

ldlA7-SRBI (Acton, 1996; Stangl, 1998): This cell line is a Chinese Hamster Ovarian (CHO) cell line that lacks LDL receptor activity (Kingsley,1984). Furthermore, ldlA7-SRBI cells overexpress the SR-BI receptor. ldlA7-SRBI cells were maintained in DHG medium, a 1:1 mixture of Dulbecco's minimal essential medium (DMEM) and Ham's F-12 medium (Gibco). The DHG medium is supplemented with 1% penicillin (100 units/ml)/streptomycin (100 mg/ml; pen/strep) and 5% fetal calf serum (FCS).

HepG2 (HB-8065; LGC Standards): These cells are human hepato-carcinoma cells and the cell line is a widely used model for human hepatocytes.
HepG2 cells were maintained in minimal essential medium (MEM; Gibco) supplemented with 1% pen/strep, 10% FCS, 1% non-essential amino acids (AA) and 1% L-glutamine.

THP-1 (TIB-202; LGC Standards): THP-1 cells are monocytic leukemia cells. It is a human monocytic cell line widely used to study foam cell formation in the development of atherosclerosis. The monocytes can be differentiated into macrophages by stimulation with phorbol esters such as PMA. THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI; PAA Laboratories) medium supplemented with 10% FCS, 1% pen/strep and 1% L-glutamine.

THP-1 differentiation

Reagents:
810 µM Phorbol-12-myristat,13-acetat, (stock solution), PMA (Sigma)
THP-1 cells, seeded at a concentration of $5*10^5$ cells/well in a 24-well plate, were differentiated in RPMI medium (supplemented with 10% FCS, 1% pen/strep and 1% L-glutamine) containing 160 nM PMA for 3 days.

3.2 **Bradford protein assay** (Bradford, 1976)

Reagents:

Bradford protein assay (BioRad Laboratories)

Albumin, bovine serum, BSA; (Sigma-Aldrich)

The Bradford assay is used to measure protein concentrations of cell lysates as well as lipoproteins. The method is based on the different absorption rates of Coomassie brilliant blue. Proteins bind to the red cationic form of Coomassie and by binding the protein-Coomassie-complex turns into the anionic form with an absorbance at 595 nm. This absorption is measured by photometry. For every assay a standard curve with several dilutions of BSA standard protein (c = 1 mg/ml) was prepared and measured together with the samples.

In micro titer plates 0, 1, 2, 5 or 10 µg BSA were mixed with 20 µl Bradford reagent and filled to a volume of 100 µl with distilled water. Similarly, 10-20 µl samples (depending on the expected concentration) were combined with the Bradford reagent and distilled water to obtain a final volume of 100 µl per well. Afterwards the absorption was measured spectrophotometrically at 595 nm using a micro plate reader (Bio-Rad, Model 550).

3.3 Lipoprotein Preparation

Reagents:

Potassium bromide, KBr (Merck)

Dialysis buffer (10x)

15 M NaCl

0,5 M EDTA

pH 7,4

Human plasma was collected from healthy, young, male and female, normal-lipidemic volunteers after an overnight fasting period. After blood sample collection, the anti-coagulant EDTA (2 ml 0,18 M EDTA per 50 ml blood sample) was added the blood and centrifuged at 3000 RPM for 20 minutes in an Eppendorf centrifuge to obtain plasma. The supernatant was re-centrifuged (at 3000 RPM for 20 minutes) to remove all red blood cells.

The density (D) of the plasma was adjusted to the corresponding specific densities of the lipoproteins with potassium bromide (KBr) to get the lipoprotein fraction.

$$D_{Plasma} = 1{,}006;\ D_{VLDL} = 1{,}019;\ D_{LDL} = 1{,}063;\ D_{HDL} = 1{,}210\ [g/ml]$$

The following formula was used to calculate the amount of potassium bromide (KBr) needed to adjust the density:

$$g\ KBr = \frac{Volume_{sample}\ (ml) * (final\ density - initial\ density)}{1 - (0{,}312 * final\ density)}$$

First, plasma was adjusted to the density of VLDL (1,019 g/ml) by KBr. Afterwards ultracentrifuge-tubes were filled with the plasma and centrifuged at 51 kRPM at 4°C for 20 h in a Beckmann ultracentrifuge (using the 55.2 rotor). The VLDL-containing plasma was removed, and the bottom fraction was adjusted to the density of LDL (1,063 g/ml), followed by another ultracentrifugation step and LDL removal. Finally, the remaining plasma was adjusted to the density of 1,21 g/ml by KBr and re-centrifuged to obtain the HDL

fraction. All lipoprotein fractions were dialyzed three times against a dialysis buffer, and protein concentrations of the lipoproteins were estimated using the Bradford protein assay.

3.4 Lipoprotein Labelling

Reagents:

Iodine^{125} 185 MBq (5 mCi, specific activity ~ 17Ci (629GBq)/mg; Hartmann Analytic)

IODO-BEADS iodination reagent kit (Pierce)

Lipoproteins were iodinated with sodium iodide^{125} (Hartmann Analytic) to measure their cellular association.

Lipoproteins were labeled using the Pierce IODO-BEADS iodination reagent kit. For every labeling procedure lipoproteins (500 µg HDL or modified HDL) were diluted in PBS to a final concentration of 1 mg/ml. The iodo beads were washed in PBS and added to the lipoprotein-PBS solutions. For each labeling procedure one iodo bead was used. Finally, the reaction was started by adding 1 mCi sodium iodide^{125}. Every sample was incubated at room temperature for 20 minutes in a radiation protected area. After incubation the reaction was stopped by removal of the iodo bead.

Lipoproteins were separated from free sodium iodide^{125} by gel chromatography using a Pierce D-Salt Dextran Desalting Column. First, the column was equilibrated with a fivefold column volume (25 ml) of PBS. The iodinated lipoprotein-solution was applied on the gel. Furthermore, the lipoprotein was eluted in five steps with PBS, first 750 µl and then with four times 500 µl. The fractions were collected and to measure incorporated radioactivity, 1 µl solution was measured in a gamma-counter (Cobra II, Packard). In a final step the protein concentration was measured using the Bradford protein assay. The labeling procedure resulted in a specific activity of ^{125}I HDL of ~ 1000 cpm/ng protein. For experiments, lipoproteins were diluted to a specific activity of ~ 300 cpm/ng protein.

3.5 High density lipoprotein modification/oxidation

Reagents:

NICK- column (Sephadex G-50, Amersham Biosciences)

2 mM $CuSO_4$ (Österreichische Apothekerkammer)

1 mM Ethylenediaminetetraacetic acid, EDTA (Merck)

5 mM Diethylenetriaminepentaacetic acid, DTPA (Merck)

Sodium hypochlorite solution, NaOCl; 12% Cl (Roth)

Potassium cyanate, KCNO (Sigma-Aldrich)

Phosphate buffer (50x stock solution, pH 7,5)

1 M Na_2HPO_4 (Merck)

1,5 M NaH_2PO_4 (Merck)

pH 7,5

Wash Buffer

50 mM TRIS

1 M Tris (hydroxymethyl) aminomethan, *TRIS* (Merck)

1,5 M NaCl

pH 7,4

$TRIS^+$-buffer

TRIS + BSA (2mg/ml)

HDL oxidation with copper modifies the lipid content of HDL particles. HDL was modified using different methods using copper ($CuSO_4$), potassium cyanate (KCNO) or hypochlorite (HOCl).

HDL modification with $CuSO_4$

First, HDL at a concentration of 4 mg/ml was separated through a NICK-column (Sephadex G-50, Amersham Biosciences) to remove EDTA, which inhibits $CuSO_4$ by complex- building (100 µl to column, rinse with 400 µl PBS, eluate with 400 µl PBS). Afterwards, HDL was incubated with $CuSO_4$ (final concentration 20µM) at 37°C for 4 hours. To stop the reaction of $CuSO_4$ with HDL EDTA (final concentration 50 µM) was added.

HDL modification with KCNO/Carbamylation

HDL at a concentration of 4 mg/ml and 20 mg/ml KCNO in phosphate buffer (50mM) was incubated at 37°Cfor 4 hours. After incubation KCNO was removed

using a NICK-column (100 µl to column, rinse with 400 µl PBS and then eluate with 400 µl PBS).

Measurement of the concentration of sodium hypochlorite (NaOCl) each month
First, *NaOCl* was mixed with 50 ml NaOH (10mM). To create dilution series (1:2) from 1:200 up to 1:1600 NaOH and *NaOCl* were mixed in adequate amounts.
The samples were measured photometrically at a wavelength at 292 nm (UV/VIS spectrometer Lambda 2, Perkin Elmer). The concentration of HOCl was determined using the molar absorption coefficient of HOCl (ε= 350 $mol.l^{-1}.cm^{-1}$).

HDL modification with HOCl (Bergt, 2004)
4 mg/ml of HDL was incubated at 37°C for 1 hour with HOCl (final concentration 4 mM) to oxidize the protein fraction of HDL. To avoid lipid oxidation DTPA (final concentration 100 µM) was added.
The reaction of HDL with HOCl was stopped using phosphate buffer (0,1 M; pH 7.5) with a final 1:2 dilution.
Modified HDL was characterized by measuring lipid peroxidation by TBARS assay and by analysis of electrophoretic mobility by gel electrophoresis.

3.6 TBARS assay (Buege and Aust, 1978)

Reagents:
Thiobarbituric acid, TBA (Sigma-Aldrich)
1 M NaOH (Merck)
1 mM butylhydroxytoluene, BHT (Merck)
1 mM EDTA (Merck)

Principle

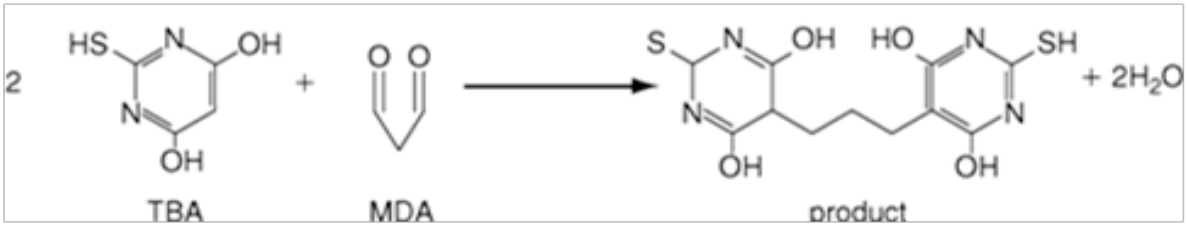
Thiobarbituric acid reactive substances (TBARS) develop during lipid peroxidation. TBARS are aledehyde breakdown products from polyunsaturated fatty acids. Malondialdehyde (MDA) is the main component of these products. One MDA molecule reacts with two molecules of thiobarbituric acid (Fig. 6) to a

pink chromogen with absorption at a maximum of 534 nm with a molar absorption coefficient of 156 000 $mol.l^{-1}.cm^{-1}$.

Figure 6. Reaction of Malondialdehyde with Thiobarbituric Acid

(http://media.wiley.com/CurrentProtocols/NS/ns0717/ns0717-fig-0009-1-full.gif; 20.07.2010)

One MDA molecule reacts with two molecules of thiobarbituric acid. The resulting product can be measured photometrically at 534 nm.



Thiobarbituric acid 1% (TBA)

0,5 g TBA was mixed with 2,5 ml NaOH (1 M). Afterwards, 47,5 ml of distilled water was added slowly to obtain 1% TBA. TBA can be stored at 4°C for at least two weeks.

For the TBARS assay 0,5 ml TBA (1%), 0,5 ml acetic acid and 0,5 ml of each sample were mixed, 10 µl BHT (10 mM) and 10 µl EDTA (1 mM) were added to avoid lipid peroxidation during incubation. Afterwards, the samples were incubated at 100°C for 45 minutes. After cooling, the samples were measured spectrophotometrically at a wavelength of 534 nm and for baseline correction at 620 nm too (UV/VIS spectrometer Lambda 2, Perkin Elmer). The molar concentration of MDA of the samples was calculated (absorption coefficient 156 000 $mol.l^{-1}.cm^{-1}$), with the Lambert- Beer's law.

The following formula calculates the molar concentration of MDA:

$$(E_{534nm} - E_{620nm}) / 156\ 000\ mol.l^{-1}.cm^{-1} * (volume_{total} / volume_{sample})$$

3.7 **Gel Electrophoresis** (Ogden and Adams, 1987)

Reagents:

Agarose (FMC Bio Products)

TAE (Tris-acetate-EDTA; 50x stock)

2 M Tris-Base

57,1 ml acetic acid

100 ml EDTA (0,5 M) pH 7,4

DNA loading dye (6x) (Fermentas)

Coomassie brilliant blue R 250 (Merck)

100% acetic acid (ProLab)

Coomassie stock solution (0,2%):

0,4 g coomassie blue was dissolved in 80 ml distilled water and 120 ml methanol was added.

Coomassie solution (0,1%):

50 ml stock solution was filtered and mixed with 10 ml acetic acid and 40 ml distilled water.

Destain solution:

20% Methanol (Fisher Scientific)

10% acetic acid (Prolab)

70% distilled water

HDL and modified HDL were separated by gel electrophoresis using 1% agarose. To prepare the gel 0,5 g agarose (FMC Bio Products) was mixed with 50 ml of TAE buffer. The mixture was heated in a microwave oven. The warm solution was applied to the casting tray and the comb was inserted. Meanwhile, 20 µg of HDL or modified HDL were mixed with a 6 x loading dye (1:6). The gel was put into the electrophoresis chamber and the chamber was filled with TAE. HDL or modified HDL containing the loading dye was load on to the gel. Electrophoresis was performed at a constant voltage at 90 V for 2 hours. The gel was stained using 0,1% coomassie solution for 15 minutes and destained

over night by destain solution containing 20% methanol, 10% acetic acid and 70% distilled water.

3.8 Cell Association Experiments

First, THP-1 cells were differentiated with 160 nM PMA in a 24-well plate at a concentration of $5*10^5$ cells/well for 3 days. After differentiation THP-1 macrophages were grown for 2 days. Additionally, HepG2 cells were plated at a concentration of $3*10^5$ cells/well and ldlA7-SRBI cells at a concentration of $1*10^5$ cells/well in 12-well plates and grown for 2 days.

On day 2 cells were washed twice with sterile PBS, and reefed with DHG medium containing 5% lpds (lipoprotein deficient serum) for ldlA7-SRBI cells, MEM + 10% lpds for HepG2 cells and RPMI.

On the experimental day (day 3) cells were washed twice with PBS (37°C). Afterwards, 0,5 ml medium (DHG + 2 mg/ml fatty acid free (faf) BSA) was added. To calculate unspecific binding a 40-fold excess of unlabeled lipoprotein (HDL) was added to every fourth well. Besides, association experiments were also performed using a 20-fold excess of unlabeled differently oxidized or modified HDL (Cu-ox. HDL, HOCl-ox. HDL or KCNO-carb. HDL). Afterwards, iodinated HDL or modified/oxidized HDL in an amount of 10 µg/ml was added to every well and 10 µl aliquots were collected to measure the specific radioactivity using a gamma-counter. After having incubated the cells for 3 hours at 37°C, the medium was removed and cells were washed twice with 50 mM ice-cold TRIS buffer and twice with TRIS + BSA (2 mg/ml). To lyse the cells 1 ml NaOH (0,1 M) was added and incubated at room temperature for 1 hour. 300 µl of the cell lysates were collected to analyze their radioactivity and counted in a gamma counter. Finally, the amount of the proteins in the cell lysates was measured by the Bradford protein assay.

The following formula calculates specific binding of proteins (ng lipoprotein/mg cell protein):

$$\frac{\text{Counts} * (\text{dilution factor/specific activity})}{\text{Total cell protein}}$$

3.9 Cholesterol Efflux Experiments

Reagents:

Cholesterol [1,2-^{3}H(N)-] (Perkin Elmer)

Scintillation cocktail – Ready Safe (Beckman Coulter)

Bovine Serum Albumin, fatty acid free, faf BSA (2 mg/ml); (PAA Laboratories)

HepG2 cells were seeded at a concentration of $1{,}5*10^{5}$ cells/well whereas ldlA7-SRBI cells were plated at a concentration of $8*10^{4}$ cells/well. THP-1 cells were seeded at a concentration of $5*10^{5}$ cells/well and differentiated with 160 nM PMA for 3 days. All cell lines were grown for 2 days. On day 2 cells were trace-labeled with 1 µCi of ^{3}H-cholesterol. On the experimental day (day 3) the cells were washed twice with PBS (37°C). Afterwards, the cells were equilibrated with medium containing faf BSA (2mg/ml) at 37°C for 2 hours. Then the supernatant was removed and the cells were incubated at 37°C up to 6 hours with HDL (10 µg/ml) or oxidized/modified HDL. Afterwards, the supernatant was centrifugated at 1750 RPM and 4°C for 10 minutes (Eppendorf Centrifuge 5403). 150 or 200 µl of the supernatant was mixed with the scintillation cocktail and counted using a beta counter (Tri-Carb 2800 TR; Perkin Elmer). Meanwhile, the cells were washed twice with PBS. 500 µl of NaOH (0,1 M) was added for cell lysis at room temperature for 1 hour. 200 or 300 µl of the cell lysates were mixed with the scintillation cocktail and counted in a beta counter. Proteins in the cell lysates were determined using the Bradford assay.

Efflux is calculated as percentage radioactivity in the media divided by the amount of radioactivity in cell lysates and media. Radioactivity was measured using a beta counter.

The following formula calculates cholesterol efflux:

$$\% \text{ Efflux} = \frac{100 \times \text{cpm (supernatant)}}{\text{cpm (media+lysates)}}$$

3.10 Quantification of Cellular Cholesterol Content by Gas Chromatography

Reagents:

FOLCH solution: chloroform: methanol (2:1)

0,1 M NaOH

The cellular lipid content was analyzed by capillary gas chromatography (GC) which allows the simultaneous quantification of free cholesterol, cholesteryl esters and triglyceride within a single GC run (Lohninger,1990).

Lipid Extraction

On day 0 cells were seeded in 6 cm dishes containing 3 ml medium. HepG2 cells at a concentration of $3*10^5$ cells per dish and ldlA7-SRBI cells at a concentration of $5*10^5$ cells per dish. THP-1 cells were seeded at a concentration of $5*10^5$ cells per dish and differentiated with 160 nM PMA for 3 days. The cells were grown for 2 days. On day 2 cells were washed once with PBS and reefed with 3 ml MEM (+ 10% lpds) for HepG2, 3 ml DHG (+ 5% lpds) for ldlA7-SRBI cells and 3 ml RPMI (+ 5% lpds) for THP-1 cells. On the experimental day (day 3) cells were kept in 3 ml DHG (HepG2 and ldlA7-SRBI cells) or 3 ml RPMI (THP-1 cells) containing 2 mg/ml faf BSA. Additionally, 10 µg/ml of native HDL, Cu- oxidized HDL, HOCl- oxidized HDL or KCNO-carbamylated HDL was added. Cells were incubated at 37°C for 3 hours. Afterwards, the cells were detached with 500 µl trypsin for 5 minutes and transferred into glass tubes with 5 ml PBS. After centrifugation at 200 g for 4 minutes the cell pellets were mixed with 1 ml of distilled water. An aliquot (50 µl) of each sample was collected and mixed with 50 µl NaOH (0,1 M) for cell lysis for 1 hour and used for protein determination by Bradford. 4 ml of FOLCH solution was added to the remaining 950 µl of each sample to start lipid extraction. The samples were mixed using a vortex mixer (Model 524, Labinco) every 5 minutes for 1 hour and centrifuged at 3000 g for 5 minutes. The

supernatant was removed and the remaining phase was concentrated under N_2 atmosphere (Liebisch equipment). The extracts were dried and dissolved in 500 µl Folch solution and an aliquot was applied to gas liquid chromatography. The analyses were carried out on the GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a programmed temperature vaporizer injector. Six-meter (0.25mm i.d.) – fused silica capillary columns with chemically bound DB-5 were used for all analyses. As internal standards tridencanoyl glycerol (for cholesterol), cholesteryl myristate (for cholesteryl esters) and trinonadecanoin (for triglycerides) were used. The chromatograms were analyzed using GC Solutions 2.3 (Shimadzu). Lipid content was normalized for cell protein and expressed in µg lipid/mg cell protein.

4 Results and Discussion

High density lipoprotein (HDL) is known to exert cardio-protective effects by promoting reverse cholesterol transport (RCT). In RCT HDL removes excess cholesterol from peripheral tissues and transports it back to liver for excretion by bile (Rader., 2008).

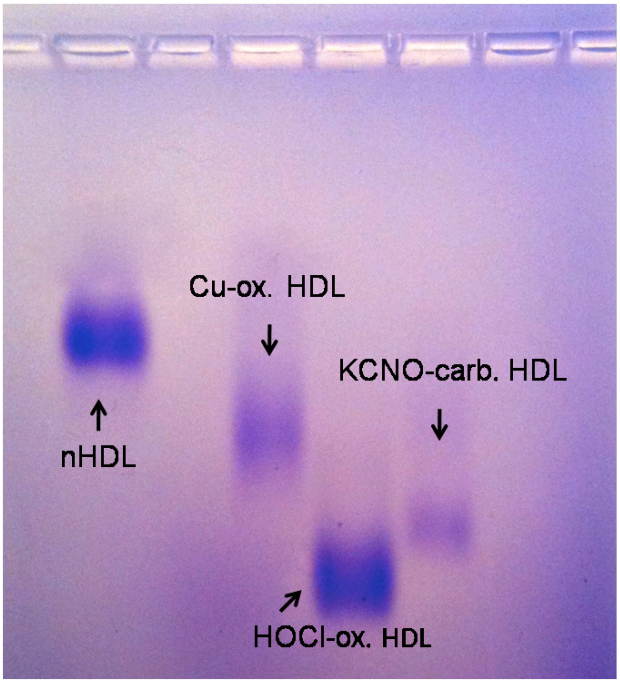
Dysfunctional HDL is implicated to play a key-role in the pathogenesis of atherosclerosis. The underlying pathways remain poorly understood. One mechanism involves oxidative modifications of apolipoprotein A-1 (apoA-1), the major protein of high density lipoprotein (Shao, 2010).

This project aims to find out if oxidation of high density lipoprotein alters the cholesterol efflux from mammalian cells. Therefore, HDL was modified with copper, hypochlorite or potassium cyanate.

First, we controlled the effectiveness of different HDL modifications by gel electrophoresis. Figure 7. shows the characterization of modified particles in terms of their electrophoretic mobility compared to native HDL. Modified HDL particles migrated more rapidly in the gel than native HDL. These results confirmed lipoprotein modifications of the apolipoprotein part. Electrophoretic mobility was HOCl-ox. HDL > KCNO- carb. HDL> Cu-ox. HDL > HDL.

Figure 7. Gel Electrophoresis

HDL was separated by gel electrophoresis using a 1% agarose gel. 20 µg of native HDL, Cu-ox. HDL, HOCl-ox. HDL or KCNO-carb. were used to perform gel electrophoresis. Electrophoresis was performed at a constant voltage of 90 V for 2 hours.



Next, the TBARS assay was applied to estimate the extent of lipid oxidation.

	Native HDL	**Cu-ox. HDL**	**HOCL-ox.HDL**	**KCNO-carb. HDL**
µM MDA	4,6 ± 0,1	10,8 ± 1,3	2,5 ± 2,7	1,4 ± 1,1

The data represent mean ± SD of five independent measurements.
The results are expressed in µg MDA (Malondialdehyde) developing in the samples during incubation time of one hour.

HDL oxidation by copper strongly induced lipid peroxidation. This resulted in an increase of malondialdehyde in copper oxidized HDL. TBARS formation in copper oxidized HDL was elevated 2-fold compared to native HDL(4,6 ± 1 vs. 10,8 ± 1,3).
In contrast, native HDL, KCNO- carbamylated HDL and HOCl- modified HDL showed no increase in TBARS formation.
To summarize, copper induced oxidation modifies the lipid content of HDL as well as its apolipoproteins, whereas hypochlorite or potassium cyanate seems to modify only the apolipoprotein content of the HDL particles.

4.1 Cholesterol Efflux in mammalian Cells

To determine cholesterol efflux from mammalian cells efflux experiments in HepG2 and ldlA7-SRBI cells up to four hours and in human THP-1 macrophages up to six hours were performed.

HepG2 cells were used as a liver model, the ldlA7-SRBI cell line as a model over-expressing the HDL receptor SR-BI and human THP-1 macrophages as a more physiological model for macrophages involved in all stages of atherosclerosis (Boyle, 2005).

4.1.1 Cholesterol Efflux in HepG2 cells

In HepG2 cells, cholesterol efflux to copper-oxidized HDL (Cu - ox. HDL), HDL modified with hypochlorite (HOCl) or HDL carbamylated by potassium cyanate (KCNO-carb.HDL) decreased significantly compared to native HDL. Cholesterol efflux was reduced compared to native HDL in a time dependent manner up to 4 hours.

It is described that hepatocytes show high levels of ABCA1 expression to promote apoA-1 mediated cholesterol efflux. In $ABCA1^{-/-}$ mice, hepatocytes showed a decrease in cholesterol efflux to HDL containing apoA-1. However, nascent apoA-1 particles are the preferred substrate for ABCA1 (Sahoo, 2004).

Malondialdehyde developed from lipid peroxidation impairs the ability of apoA-1 to interfere with ABCA1 which results in decreased cholesterol efflux (Shao, 2010).

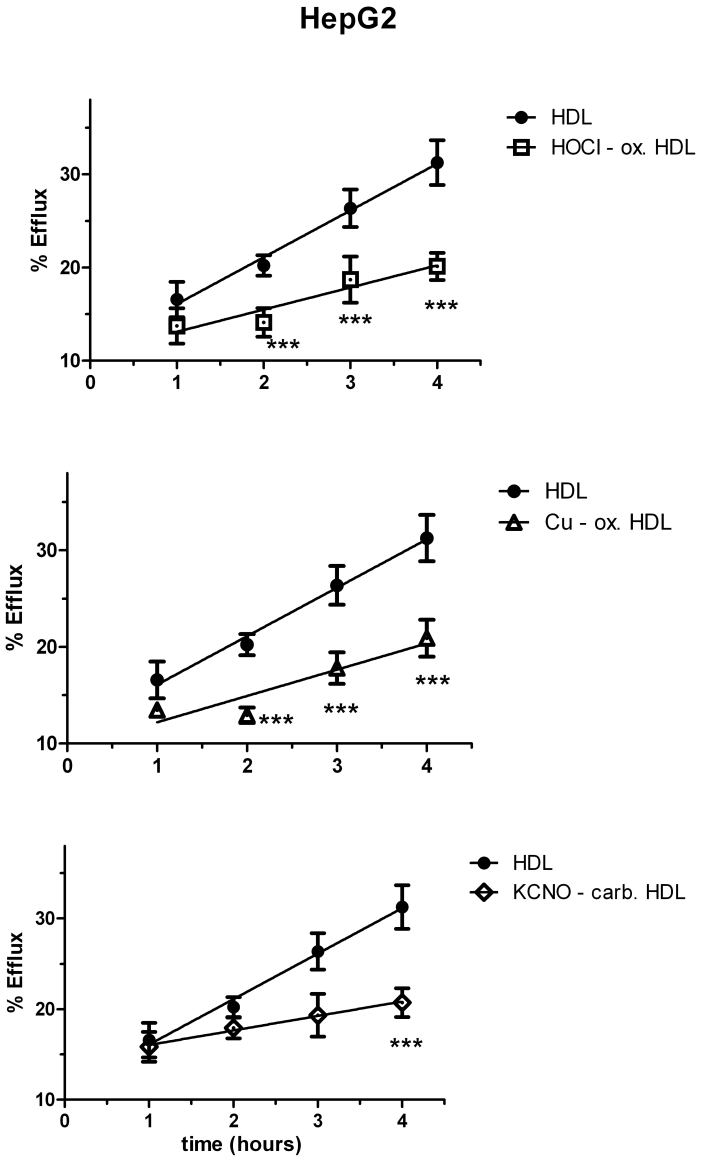
Bergt et al. (2004) showed that HDL exposed to HOCl was less able to remove cholesterol from cultured cells. The ABCA1 pathway may also be impaired by dysfunctional HDL (Bergt, 2004; Shao, 2010).

These findings, together with our results, indicate that all modifications tested alter efflux to HDL. Consequently, different receptors including SR-BI and ABCG1 (which are expressed in HepG2 cells) might be involved in the effects observed.

Taken together, cholesterol efflux decreased ~ 70% within four hours using HOCl-ox. HDL or Cu-ox. HDL in HepG2 cells. KCNO-carb. HDL exhibited a reduction of cholesterol efflux of ~ 60% after 4 hours of incubation.

Figure 8. Cholesterol Efflux in HepG2 cells

*HepG2 cells were seeded into 24-well plates at a density of 1,5*10^5 cells/well. Cells were grown for 2 days followed by trace-labeling with 1 µCi of ^{3}H-cholesterol. On day 3 cells were washed twice with PBS and equilibrated with MEM containing faf-BSA (2 mg/ml) at 37°C for 2 hours. After 2 hours the supernatant was removed and efflux at 37°C up to 4 hours was started. Therefore cells were incubated with 10 µg/ml of HDL, Cu-ox. HDL, HOCl-ox.HDL or KCNO-carb.HDL. Afterwards, 150 or 200 µl of the supernatant were mixed with the scintillation cocktail and counted in a beta counter*



Each data point represents mean of triplicates of two independent experiments. The results are expressed as % of initial loading (100%). Statistical significance

was analyzed using an unpaired t-test for all experiments. P < 0,001 (***); r^2 > 0,97

4.1.2 Cholesterol Efflux in ldlA7-SRBI cells

In the ldlA7-SRBI cell line copper-induced modification of HDL decreased cholesterol efflux significantly up to 4 hours. The data points show a linear regression of r^2 > 0,97, this may indicate further reduction of cholesterol efflux beyond an incubation time of 4 hours (Fig. 10).

Also HDL modified by hypochlorite showed a decrease of cholesterol efflux, especially after 4 hours, efflux dropped to 75% compared to native HDL, which serves as a control. It is well established that myeloperoxidase (MPO) is involved *in vivo* in generating oxidants which are associated risk factors in atherogenesis. Hypochlorite modified HDL is widely used as a model for myeloperoxidase products occurring in atherosclerotic lesions (Marsche, 2008). All the more it was interesting to estimate the influence of HOCl-modified HDL on cholesterol efflux.

In ldlA7-SRBI cells there was also a trend to decrease efflux for KCNO-carb.HDL, although these effects were not pronounced and did not reach statistical significance.

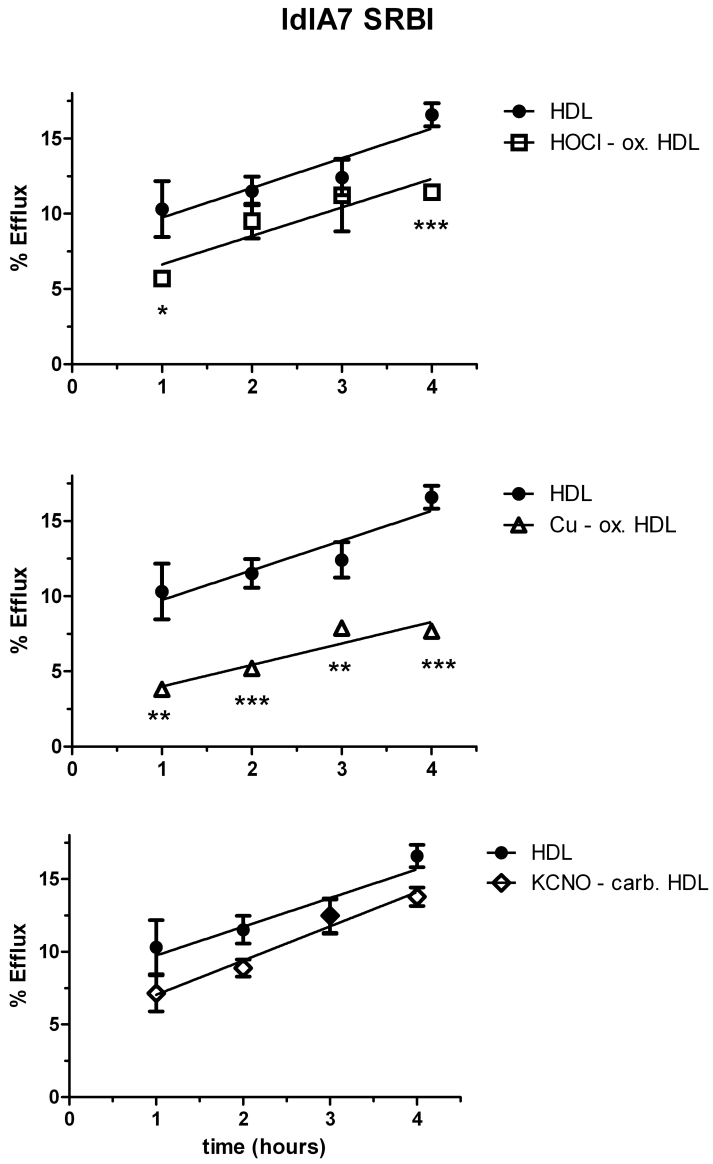
SR-BI is reported to serve as a ligand for oxidized HDL as well as HDL (Marsche, 2002).

Especially, Cu-oxidized HDL resulted in a ~ 50% reduction of cholesterol efflux compared to native HDL.

Figure 9. Influence of modified HDL on cholesterol efflux from ldlA7-SRBI cells.

Cells were grown and treated as described in Figure 9 (HepG2). ldlA7-SRBI cells were plated in 24-well plates at a concentration of $8*10^4$ cells/well.

Efflux was calculated as percentage radioactivity in the media divided by the amount of radioactivity from cell lysates and media.100% represents the initial loading of the cells.



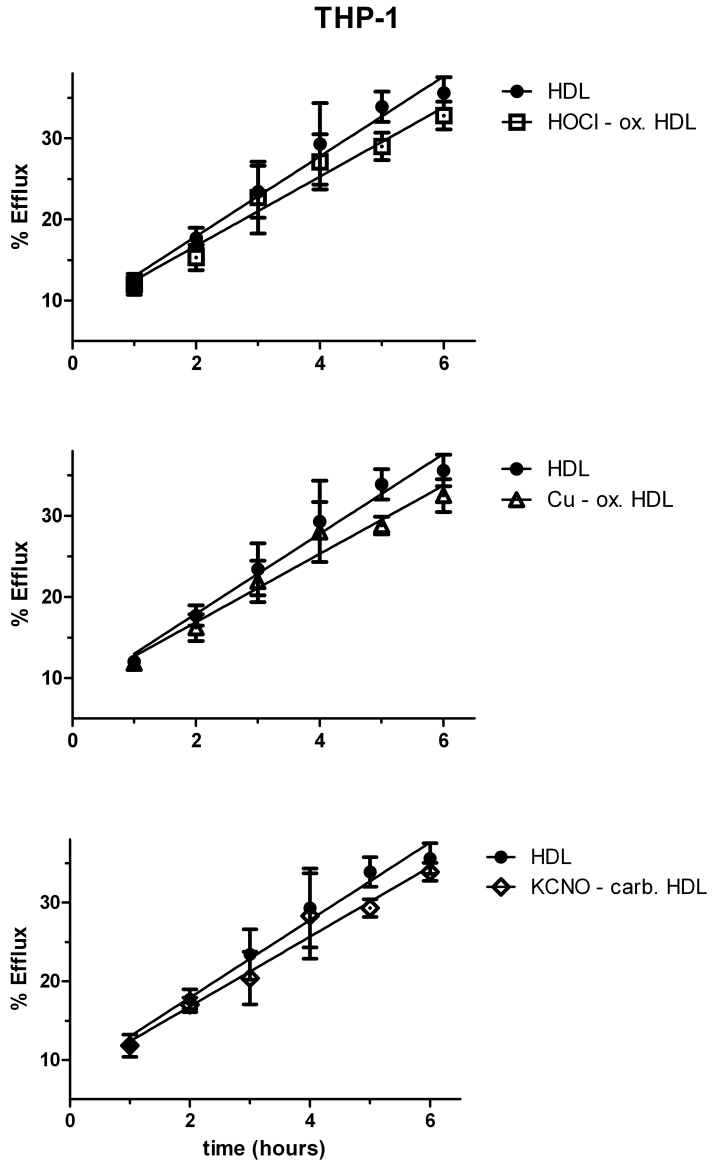
Each data point represents triplicates of one representative experiment. The results are expressed in % of initial loading of the cells.
$p < 0,05$(*); $p < 0,01$ (**); $p < 0,001$ (***); linear regression: $r^2 > 0,97$

4.1.3 Cholesterol Efflux in THP-1 Macrophages

Macrophages are involved in all stages in the pathogenesis of atherosclerosis (Corrado, 2010). Nevertheless, THP-1 macrophages showed almost no response to different modified HDL. After 6 hours incubation with copper-oxidized HDL (Cu - ox. HDL), HDL modified with hypochlorite (HOCl) or HDL carbamylated by potassium cyanate (KCNO-carb.HDL) we determined a tendency of reduced cholesterol efflux.

Figure 10. Cholesterol Efflux to modified HDL in THP-1 macrophages

Human THP-1 monocytes were differentiated into macrophages over 3 days with 160 nM PMA at a concentration of $5*10^5$ cells/well. After differentiation cells were depleted with 5% lpds and trace-labeled using 1 µCi of ^{3}H-cholesterol as described in Figure 9 (HepG2). To analyze cholesterol efflux cells were incubated with 10 µg/ml of native HDL or HDL modified by copper, hypochlorite or potassium cyanate.



Each data point represents mean ± SD of triplicates of three independent experiments. The results are expressed as % of initial loading. Statistical significance was analyzed using an unpaired t-test for all experiments. Linear regression $r^2 > 0,98$

4.2 Gas Chromatography Analysis of Cellular Cholesterol Content

We wanted to confirm, whether decrease in cholesterol efflux to modified HDL results in increased cellular cholesterol levels. To quantify cellular cholesterol content we performed gas – liquid chromatography in HepG2 and ldlA7-SRBI cells.

4.2.1 Cellular Cholesterol Content in HepG2 cells

	control	**HDL**	**Cu-ox. HDL**	**HOCl-ox. HDL**	**KCNO-carb. HDL**
CE	26,8 ± 8,0	23,7 ± 8,5	30,2 ± 20,9	33,1 ± 19,4	40,2 ± 35,4
FC	130,7 ± 18,2	108,6 ± 13,4	119,3 ± 38,9	145,9 ± 46,6	146,5 ± 58,4
TC	157,5 ± 25,7	132,3 ± 20,1	149,5 ± 58,9	179,0 ± 66,6	186,7 ± 93,5

The results are expressed as µg of CE, FC or TC/mg cell protein. The data represent mean ± SD of four independent experiments.

First, we estimated free cholesterol (FC), cholesteryl esters (CE) and total cholesterol (TC) in HepG2 cells after 3 hour incubation with HDL or differently modified HDL (10 µg/ml). In liver cells CE are stored in a high amount (Maxfield, 2002). Our results show that HepG2 cells had a higher content of CE than ldlA7-SRBI cells. For example, incubation with native HDL results in a CE content of ~ 24 µg /mg protein in HepG 2 cells (Fig. 12); in contrast in ldlA7-SRBI cells (Fig. 13) only ~ 1,2 µg CE/ mg protein were detectable.

In HepG2 cells, neither, incubation with Cu-oxidized HDL or HOCl-oxidized HDL, nor, KCNO-carb. HDL showed a significant change in CE, FC and TC content. Nevertheless, differently modified HDLs showed an increase of cellular cholesterol content compared to native HDL. However, intra-experimental deviations were too large to reach statistical significance. This leads to the assumption, that modification of HDL induced by HOCl or KCNO may tend to result in an increase of CE, FC and TC.

Taken together, these data confirm our findings for efflux studies: modified HDL cause reduced cholesterol efflux and therefore cellular lipid contents were increased.

4.2.2 Cellular Cholesterol Content in ldlA7-SRBI cells

	Control	**HDL**	**Cu-ox. HDL**	**HOCl-ox. HDL**	**KCNO-carb. HDL**
CE	0,3 ± 0,3	1,4 ± 0,3	1,4 ± 0,3	2,6 ± 0,5	2,1 ± 0,3
FC	40,0 ± 3,4	42,8 ± 2,3	46,4 ± 1,6	47,0 ± 0,6	46,7 ± 1,2
TC	40,3 ± 3,5	44,3 ± 4,0	47,8 ± 1,3	49,7 ± 1,0	48,9 ± 1,1

The results are expressed as µg of CE, FC or TC/mg cell protein. The data represent mean ± SD of triplicates of four independent experiments.

In the ldlA7 SR-BI cell line we observed a significant increase of CE derived from HOCl-oxidized HDL compared to native HDL. Modification of HDL by HOCl significantly increased CE within cells from 1,4 to 2,6 µg/mg cell protein; in other words CE increased about ~ 50% compared to native HDL. KCNO-induced carbamylation of HDL, similarly to HOCl-ox. HDL, showed a 50% increase from 1,4 to 2,1 µg/mg cell protein, whereas copper induced oxidation of HDL showed no significant increase compared to native HDL.

Free cholesterol was estimated in an average amount of 40 µg/mg protein (after incubation with or without HDL). In analogy to cholesteryl esters (CE), free cholesterol (FC) significantly increased due to modifications of HDL by hypochlorite or KCNO, again almost no difference to native HDL came up by incubation of ldlA7 cells with copper-oxidized HDL. FC content just increased slightly from ~ 40 to ~ 47 µg/ mg cell protein. Furthermore, we estimated total cholesterol (TC) and observed no significant change in the total cholesterol content by incubation with modified HDL compared to native HDL in ldlA7-SRBI cells. These data confirm in some extent our findings from the efflux studies: HOCl-ox. HDL and KCNO-carb. HDL has reduced efflux and therefore lipid content was increased by these modifications in ldlA7-SRBI cells. Although, Cu-ox. HDL reduced cholesterol efflux about ~ 50% compared to native HDL, there was almost no increase of cellular lipids induced by Cu-ox. HDL.

4.3 Cell Association of [^{125}I]-HDL and [^{125}I]-modified HDL

To analyze if modifications of HDL result in different cell binding and uptake we performed association and competition experiments in HepG2, ldlA7-SRBI cells and THP-1 macrophages.

4.3.1 Cell Association in HepG2 cells

	Total Binding	**Specific Binding (20x lipoprotein)**	**Specific Binding (40x HDL)**
HDL	147,3	79,0	29,0
HOCl-ox. HDL	97,0	38,5	41,0
Cu-ox. HDL	119,0	63,0	35,5
KCNO-carb. HDL	146,3	90,0	63,0

The results are expressed as ng lipoprotein/mg cell protein. The data represent means of triplicates of one representative experiment.

In HepG2 cells no effect of native HDL (40-fold excess) on ^{125}I HDL or ^{125}I HDL modified by Cu^{2+} or HOCl in regard to specific binding was observed. ^{125}I KCNO-carbamylated HDL showed a slightly increase in specific binding competed by native HDL (40-fold excess). We observed nearly the same results on specific binding of ^{125}I HDL or ^{125}I modified HDL competed by HDL, Cu-ox. HDL, HOCL-ox. HDL or KCNO-carb. HDL (20-fold excess of each).

Compared to ldlA7 SR-BI over-expression cells HDL cell association in HepG2 cells was reduced to ~ 80%. HepG2 cells contain few SR-BI, known as a ligand for HDL, compared to ldlA7-SRBI cells. This could be the reason for reduced HDL cell association of HDL.

4.3.2 Cell Association in ldlA7-SRBI cells

	Total Binding	**Specific Binding (20x lipoprotein)**	**Specific Binding (40x HDL)**
HDL	263,5	107,3	86,0

HOCl-ox. HDL	225,5	142,5	162,0
Cu-ox. HDL	346,2	191,7	182,7
KCNO-carb. HDL	124	61,0	44,0

The data represent means of triplicates of two independent experiments. The results are expressed as ng lipoprotein/mg cell protein.

Specific cell association, termed as competition of ^{125}I HDL or ^{125}I modified HDL with 20x excess of unlabeled native or modified HDL, was nearly the same as competition by native HDL in ldlA7-SRBI cells. Only KCNO-carbamylated HDL competed well with native HDL in a 40-fold excess. Nevertheless, the results clearly demonstrate an increase of cell association induced by Cu2+ oxidized HDL compared to native HDL by ~ 31%. SR-BI, is assumed to be a multiligand receptor to bind oxidized HDL as well as native HDL, LDL or oxidized HDL and mediated selective uptake of CE. This may indicate a high affinity of modified HDL to SR-BI promoting selective CE uptake. Our results may indicate a high affinity of SR-BI to oxidized HDL.

4.3.3 Cell Association in THP-1 Macrophages

	Total Binding	**Specific Binding (20x lipoprotein)**	**Specific Binding (40x HDL)**
HDL	118,0	68,0	7
HOCl-ox. HDL	66,7	49,0	31,5
Cu-ox. HDL	259,0	142,5	209,3
KCNO-carb. HDL	72,3	33,5	61,5

The results are expressed as ng lipoprotein/mg cell protein. The data represent means of triplicates of two independent experiments.

THP-1 macrophages revealed a 2-fold increase of Cu-ox. HDL cell association compared to HDL cell association. Nevertheless, Cu-ox. HDL was not well competed by native HDL. This was also reported by Nakajima et al. (2000). Taken together, these data indicate that all tested modifications of HDL result in a different receptor affinity compared to native HDL.

5 Conclusion

Dysfunctional high density lipoprotein (HDL), mainly generated through modifications of the major protein of HDL, apoA-1, is assumed to be implicated in the pathogenesis of atherosclerosis (Shao, 2010). It is thought to alter reverse cholesterol transport (RCT), an important process in the removal of excess cholesterol from cells for transport to the liver for disposal into bile. Furthermore, dysfunctional HDL is supposed to reduce cholesterol efflux from cells (Marsche, 2002).

We detected a cell-specific response to modified HDL in tissue culture cells. Modification of HDL by copper, hypochlorite or potassium cyanate strongly induced a decrease of cholesterol efflux capacities in HepG2 cells.

In ldlA7 SR-BI cells modifications of HDL by copper and hypochlorite resulted in a time-dependent decrease of cholesterol efflux. THP-1 macrophages showed almost no response to differently modified HDLs.

The alterations in cholesterol efflux capacities resulted in increased lipid content in HepG2 and ldlA7-SRBI cells. We assumed that these effects were in part the results of different receptor binding as a consequence of HDL modifications. These effects differed for the single modifications.

Taken together, we showed altered cholesterol efflux in tissue culture cell lines. Moreover, these effects might also modulate RCT *in vivo* and might be a mechanism for the induced atherogenity of modified HDLs.

6 Abstract

The cardioprotective effects of high density lipoprotein (HDL) are generally attributed to reverse cholesterol transport, a process to remove excess cholesterol from artery wall macrophages to liver for excretion into bile.

It has been proposed that HDL looses its cardioprotective ability through oxidative modifications by reactive oxygen species or myeloperoxidase, both of them are present in atherosclerotic lesions *in vivo*. One potential mechanism generating dysfunctional HDL involves oxidative modifications of apoA-1, the major protein of HDL.

This project aimed to characterize, whether oxidation of HDL exhibits alterations on lipid transfer from HDL to cells and vice versa. Therefore, HDL was oxidized or modified using copper, potassium cyanate or hypochlorite. The resulting oxidation products were analyzed on regard lipid oxidation (TBARS assay) and to electrophoretic mobility. We have analyzed the impact of modified HDL on cholesterol efflux in human THP-1 macrophages, human hepato-carcinoma cells (HepG2) and in Chinese hamster ovarian (CHO) cells over-expressing scavenger receptor class B type 1 (ldlA7-SRBI). HOCl- and Cu^{2+}- induced modification of HDL resulted in a significant, time–dependent decrease in ^{3}H-Cholesterol efflux from HepG2 and ldlA7-SRBI cells, whereas THP-1 macrophages exhibited almost no response to modified HDL. Decreased efflux resulted in elevated free cholesterol (FC), cholesteryl esters (CE) and total cholesterol (TC) levels, as measured by gas chromatography. Furthermore, we attempted to analyze, whether these effects were due to altered receptor interaction as a result of the modifications.

Taken together we found that modified HDL negatively affects cholesterol efflux in tissue culture cells. The generation of modified or oxidized HDL *in vivo* might therefore be regarded as possibly atherogenic.

7 Zusammenfassung

Viele Studien bestätigen die atheroprotektive Wirkung von high density lipoprotein (HDL). Diese wird der Rolle von HDL im „Reversen Cholesterin Transport“ (RCT) zugeschrieben. Dabei wird überschüssiges Cholesterin aus peripheren Zellen von HDL zur Leber transportiert und über die Galle ausgeschieden.

Es wird vermutet, dass HDL seine schützende Wirkung verliert, wenn es durch freie Radikale oder durch Enzyme mit oxidierender Wirkung, die bei entzündlichen Erkrankungen- wie auch im Fall der Atherosklerose- im Körper vermehrt freigesetzt werden, modifiziert wird.

Thema dieser Diplomarbeit war zu erforschen, inwiefern sich Modifikation/ Oxidation von HDL auf den Cholesterinefflux in Zellkultur auswirkt. Für dieses Projekt wurden folgende Zelllinien verwendet: humane Lebertumorzellen (HepG2), Zellen, die den HDL-Rezeptor SR-BI überexprimieren (ldlA7-SRBI) und humane THP-1 Makrophagen. HDL wurde einerseits durch Oxidation mittels Kupfer und Hyperchlorit, andererseits mittels Kaliumcyanat, modifiziert. Besonders in den Zelllinien HepG2 und ldlA7-SRBI wurde der Efflux von Cholesterin aus der Zelle bis zu 70% verringert. THP-1 Makrophagen zeigten in Bezug auf modifiziertes HDL kaum eine Änderung des Cholesterinefflux.

Mittels Gaschromatographie wurde der Gehalt an Cholesterin nach Behandlung mit nativem HDL und modifiziertem HDL analysiert. Die HDL Modifizierung führte zu einem erhöhten Gehalt an zellulärem Gesamtcholesterin (TC), freiem Cholesterin (FC) und Cholesterinestern (CE). Abschließend wurde untersucht, ob die Modifizierung zu einer veränderten Affinität zu zellulären Rezeptoren führt.

Zusammengefasst kann gesagt werden, dass Modifizierungen von HDL den Cholesterinefflux senken und somit modifiziertes HDL als atherogen angesehen werden muß.

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